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Structural requirements at the melatonin receptor

[1]David Sugden, [2]Nelson W.S. Chong & *David F.V. Lewis

Physiology Group, Biomedical Sciences Division, King's College London, Campden Hill Road, Kensington, London W8 7AH and *School of Biological Sciences, University of Surrey, Guildford, Surrey GU2 5XH

1 High affinity, specific binding sites for the pineal hormone, melatonin (5-methoxy *N*-acetyltryptamine) can be detected in chick brain membranes by use of the radiolabelled agonist, 2-[^{125}I]-iodomelatonin (2-[^{125}I]-aMT).

2 The affinity of a number of analogues of melatonin at the 2-[^{125}I]-aMT binding site was determined and compared with an analysis of their electronic structure and significant quantitative relationships obtained.

3 The best correlations indicated that binding affinity was correlated with ΔE, the difference between the frontier orbital energies, and Q_NH, the electron density in the highest occupied molecular orbital of the side-chain nitrogen atom.

4 These findings suggest that ligand binding may involve hydrogen bonding between the 5-methoxy and amide moieties of melatonin and complementary amino acid residues, and charge transfer interactions between the indole ring of melatonin and an aromatic amino acid in the receptor binding site.

5 A molecular model of a putative binding site is proposed based on the predicted amino acid sequence of the cloned *Xenopus laevis* melanophore melatonin receptor and the quantitative structure-affinity relationships observed in the present study.

Keywords: Melatonin receptor; 2-[^{125}I]-iodomelatonin; chick brain binding; QSAR; molecular modelling

Introduction

Melatonin (5-methoxy *N*-acetyltryptamine, aMT) is synthesized in the pineal gland of vertebrates and secreted into the general circulation only at night (Sugden, 1989). A primary role for circulating melatonin in regulating various seasonal changes in physiology, such as reproduction, metabolism and hair growth, in many photoperiodic species is well-established (Tamarkin *et al.*, 1985; Bartness & Goldman, 1989). Pineal melatonin is also involved in regulating circadian rhythms in some species of birds and reptiles (Underwood, 1989) and, low doses of exogenous melatonin entrain free-running rhythms in mammals (Redman *et al.*, 1983; Folkard *et al.*, 1990; Thorpe & Coen, 1994), while larger doses of have a sleep-promoting and sedative effect in both experimental animals and man (Holmes & Sugden, 1982; Waldhauser *et al.*, 1990). Small amounts of melatonin are also synthesized by the photoreceptors of some species where it probably acts locally to regulate various aspects of retinal physiology such as photoreceptor retinomotor movements and photoreceptor outer segment disc shedding (Besharse *et al.*, 1988).

Recent studies using 2-[^{125}I]-iodomelatonin, a ligand originally developed for use in aMT radioimmunoassay (Vakkuri *et al.*, 1984), identified and characterized specific, high affinity receptors which probably mediate at least some of these actions of melatonin (Krause & Dubocovich, 1991; Morgan *et al.*, 1994). Our studies on the high affinity 2-[^{125}I]-iodomelatonin binding sites found in several tissues (brain, pars tuberalis and retina) and species (sheep, chicken and wallaby) using a series of novel melatonin analogues have indicated a considerable similarity of melatonin receptor characteristics (Sugden & Chong, 1991; Paterson *et al.*, 1992). In the present study we have combined radioreceptor data with an analysis of the electronic structure of a number of these melatonin analogues to provide a quantitative analysis of the structure-affinity relationships at the melatonin receptor. Our studies suggest a model of melatonin binding to its receptor in which hydrogen bonding occurs between 5-methoxy and amide groups of melatonin and complementary amino acid residues at the binding site.

[1] Author for correspondence.
[2] Present address: Department of Neuroscience, Institute of Psychiatry, De Crespigny Park, Denmark Hill, London SE5 8AF.

Methods

Membrane preparation and binding experiments

Chickens (*Gallus domesticus*, White Leghorn) were obtained from Orchard Farms (Buckinghamshire) at 1 day of age and were housed under a diurnal lighting cycle (12L:12D, lights on at 06 h 00 min) until killed between 14 h 00 min and 15 h 00 min at 15 days of age. Whole brain was removed and frozen in liquid nitrogen and stored at −70°C. Brains were homogenized in 20 vol of Tris-HCl (50 mM, pH 7.4) containing phenyl methylsulphonylfluoride (PMSF) (1 mM), leupeptin (50 μg ml^{-1}) and EGTA (1 mM). The homogenate was centrifuged (100,000 *g*, 4°C, 60 min) and the pellet rehomogenized in the same buffer and spun for a second time. The final membrane pellet was resuspended in Tris-HCl and aliquots frozen at −70°C until used. Binding assays were done in duplicate by incubating 2-[^{125}I]-iodomelatonin (30 to 50 pM) with membranes (60 μg of protein) for 60 min at 25°C. Nonspecific binding was defined with cold melatonin (1 μM). Protein was determined by a dye-binding method with bovine serum albumin as the standard (Bradford, 1976).

Data analysis

Saturation experiments were analysed by non-linear regression analysis using the ENZFITTER programme (Leatherbarrow, 1987) with the equation

$$B = B_{max} * F/(K_d + F)$$

where B = the concentration of ligand bound to the receptor, F = the concentration of free ligand, K_d = the equilibrium

dissociation constant and B_{max} = the maximal concentration of binding sites. IC_{50} values were determined in competition assays using the ALLFIT programme (DeLean *et al.*, 1978) with the four parameter logistic equation

$$Y = \frac{A - D}{1 + (X/C)^B} + D$$

where X and Y are the concentration of the competing compound and percentage inhibition of 2-[^{125}I]-iodomelatonin binding respectively, and A is the maximal binding (in the absence of competitor), B is the slope factor, C is the IC_{50} and D is the minimal binding (nonspecific binding). Inhibition constants (K_i) were then calculated with the Cheng-Prusoff equation (Cheng & Prusoff, 1973).

Reagents

2-[^{125}I]-iodomelatonin (2200 Ci mmol^{-1}) was purchased from DuPont U.K. Ltd. (Stevenage, U.K.). Melatonin analogues were obtained from the following sources: 2-chloromelatonin, Dr C.E. Smithen (Roche Products, U.K.); 6-fluoromelatonin and 6-chloromelatonin, Lilly Research Labs. (Indianapolis, U.S.A.); 6-methoxymelatonin, Dr D.C. Klein (National Institutes of Health, U.S.A.); 5-methoxy *O*-acetyltryptamine, Dr I. Smith (Middlesex Hospital Medical School, U.K.). 5-Methoxy *N*-isobutanoyltryptamine, 5-methoxy *N*-valeroyltryptamine, 5-methyl *N*-acetyltryptamine, 5-benzyloxy *N*-acetyltryptamine and N-acetyltryptamine were synthesized from the free amines as described previously (Ho *et al.*, 1968) and shown to be pure after recrystallization by thin-layer chromatography on silica gel plates (Anachem, Luton, U.K.) using two systems (I: ethyl acetate/n-butanol, 90/10; II: chlorform/methanol/acetic acid/water, 60/30/3/1). The structures were confirmed by ^{1}H n.m.r. (The Bruker WM 250 NMR University of London Intercollegiate Research Service for NMR at King's College). All other compounds were purchased from Sigma Chemical Co. (Poole, Dorset, U.K.) or Aldrich Chemical Co. (Gillingham, Dorset, U.K.).

Structural analysis

The molecular geometries of the melatonin analogues were optimised to give the minimum energy conformations using a combination of Newton-Raphson and Simplex methods found in the COSMIC molecular modelling package obtained from Smith Kline and French Ltd., Welwyn Garden City, U.K. (Vinter *et al.*, 1987).

Electronic structure calculations were executed on the minimized geometries using the CNDO/2 molecular orbital method (Pople *et al.*, 1965) using COSMIC. The resulting electronic distributions were used to generate molecular electrostatic potential energies for the melatonin analogues. QSARs were produced by stepwise multiple regression analysis of the electronic structural parameters, comprising a data set of 30 independent variables, with K_i values obtained in competition experiments using 2-[^{125}I]-iodomelatonin (Table 1). Molecular modelling and quantum chemical calculations were carried out on a MicroVAX II mini-computer and statistical processing was performed on a Cyber 176 mainframe at the University of Manchester Regional Computer Centre. The putative receptor binding site model was constructed using the SYBYL molecular modelling software package (Tripos Associates, St. Louis, U.S.A.).

Results

Binding of 2-[^{125}I]-iodomelatonin to chicken brain membranes

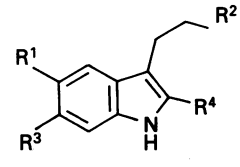
Binding of the melatonin agonist 2-[^{125}I]-iodomelatonin to chicken brain membranes was saturable and to a single population of binding sites with an equilibrium dissociation constant (K_d) of 29.4 ± 2.7 pM and a maximal density of binding sites (B_{max}) of 11.1 ± 0.4 fmól mg^{-1} protein ($n = 3$). The structures of the melatonin analogues used are shown in Figure 1. The results of the competition experiments are shown in Figure 2a and b and K_i values are presented in Table 1. All of the melatonin analogues examined displaced 2-[^{125}I]-iodomelatonin binding completely. In all cases the pseudo Hill coefficient was close to unity (0.80 to 1.10).

Substitution of chlorine in the 2-position of the indole ring of melatonin increased binding affinity, while fluorine or chlorine substitutions in the 6-position were reasonably well-tolerated. Other 6-position (methoxy, hydroxy) or 5-position substitutions (methyl, benzoyl) reduced affinity as did long side-chain acyl groups (valeroyl, isobutanoyl).

Table 1 Inhibition constants (K_i) for competition of 2-[^{125}I]-iodomelatonin (2-[^{125}I]-aMT) binding in chick brain membranes

	Compound	K_i (nM)	pK_i
1	2-Chloromelatonin	0.024 ± 0.004	−10.62
2	Melatonin	0.24 ± 0.01	−9.62
3	6-Fluoromelatonin	0.36 ± 0.04	−9.44
4	6-Chloromelatonin	0.58 ± 0.07	−9.24
5	6-Hydroxymelatonin	6.3 ± 0.4	−8.20
6	N-isobutanoyl 5-methoxytryptamine	6.1 ± 0.2	−8.21
7	N-valeroyl 5-methoxytryptamine	12.6 ± 0.4	−7.90
8	6-Methoxymelatonin	31.7 ± 2.6	−7.50
9	5-Methyl *N*-acetyltryptamine	146.8 ± 28.6	−6.83
10	5-Benzoyl *N*-acetyltryptamine	170.0 ± 6.1	−6.77
11	O-acetyl 5-methoxytryptamine	242.2 ± 24.2	−6.62
12	N-acetyltryptamine	730 ± 40	−6.14
13	N-acetyl 5-hydroxytryptamine	488 ± 14	−6.31
14	5-Methoxytryptamine	2528 ± 149	−5.60

IC_{50} values were determined in competition assays using a four parameter logistic equation on the ALLFIT programme (De Lean *et al.*, 1978) and K_i calculated using the Cheng-Prusoff equation (Cheng & Prusoff, 1973). The values given are the mean ± s.e.mean of 3 determinations. Hill coefficients were between 0.8 and 1.10.



	Compound	R_1	R_2	R_3	R_4
1	2-Chloromelatonin	OMe	NHCOMe	H	Cl
2	Melatonin	OMe	NHCOMe	H	H
3	6-Fluoromelatonin	OMe	NHCOMe	F	H
4	6-Chloromelatonin	OMe	NHCOMe	Cl	H
5	6-Hydroxymelatonin	OMe	NHCOMe	OH	H
6	N-isobutanoyl 5-methoxytryptamine	OMe	$NHCOPr^i$	H	H
7	N-valeroyl 5-methoxytryptamine	OMe	NHCOBu	H	H
8	6-Methoxymelatonin	OMe	NHCOMe	OMe	H
9	5-Methyl *N*-acetyltryptamine	Me	NHCOMe	H	H
10	5-Benzoyl *N*-acetyltryptamine	OCH_2Ph	NHCOMe	H	H
11	O-acetyl 5-methoxytryptamine	OMe	OCOMe	H	H
12	N-acetyltryptamine	H	NHCOMe	H	H
13	N-acetyl 5-hydroxytryptamine	OH	NHCOMe	H	H
14	5-Methoxytryptamine	OMe	NH_2	H	H

Figure 1 Structures of melatonin analogues used in this study

Quantitative structure-affinity analysis

A quantitative relationship between the electronic structural parameters derived from CNDO/2 calculations and the affinity of the analogues at the chicken brain 2-[^{125}I]-iodomelatonin binding site was evident. The best correlations were:-

$$pK_i = -841.1\Delta E^{-1} - 62.6$$
$$(\pm 248.4) \qquad (n = 14,\ s = 1.125,\ R = 0.70,\ F = 11.5)$$

and,

$$pK_i = 770.7\Delta E^{-1} + 51.2\ Q_NH - 58.8$$
$$(\pm 229.3) \quad (\pm 27.5)$$
$$(n = 14,\ s = 1.024,\ R = 0.78,\ F = 8.7)$$

Where n = number of points, s = standard error, R = correlation coefficient, and F = variance ratio. It can be seen from Figure 3 that melatonin (2) is an outlier having a higher affinity than would be expected, and improved correlations were obtained if melatonin was excluded from the analysis:

$$pK_i = -4.9\Delta E + 45.3\ Q_NH + 64.8$$
$$(\pm 0.9) \quad (\pm 18.8)$$
$$(n = 13,\ s = 0.699,\ R = 0.90,\ F = 21.6)$$

This suggests that for the endogenous ligand additional factors, not represented in this analysis of electronic structure, may be important determinants of binding affinity. Given the similarities in their structures it seems unlikely that melatonin could bind to the receptor in an entirely different manner to the analogues. Thus the endogenous ligand is likely to possess specific molecular characteristics not entirely identifiable in the structural descriptors employed in the QSAR analysis. As the majority of the analogues of melatonin studied possess additional substituents or bulkier side-chains than melatonin itself, it might be that the greater conformational flexibility of melatonin relative to the other analogues allows it easier entry to the binding site, or confers an enhanced ability to adopt the appropriate shape needed for interaction with key amino acid residues in the binding site.

In each of these correlations the term ΔE is apparent. ΔE is the difference between the frontier orbital energies and relates to the ability of the molecule to participate in charge transfer interactions. Inclusion of a second parameter, Q_NH, the electron density in the highest occupied molecular orbital of the side-chain nitrogen atom (oxygen in the case of 11) improves the correlation, although the variance ratio diminishes. Log P, the hydrophobic parameter, has much the same effect as a secondary descriptor but the correlation is lower (data not shown).

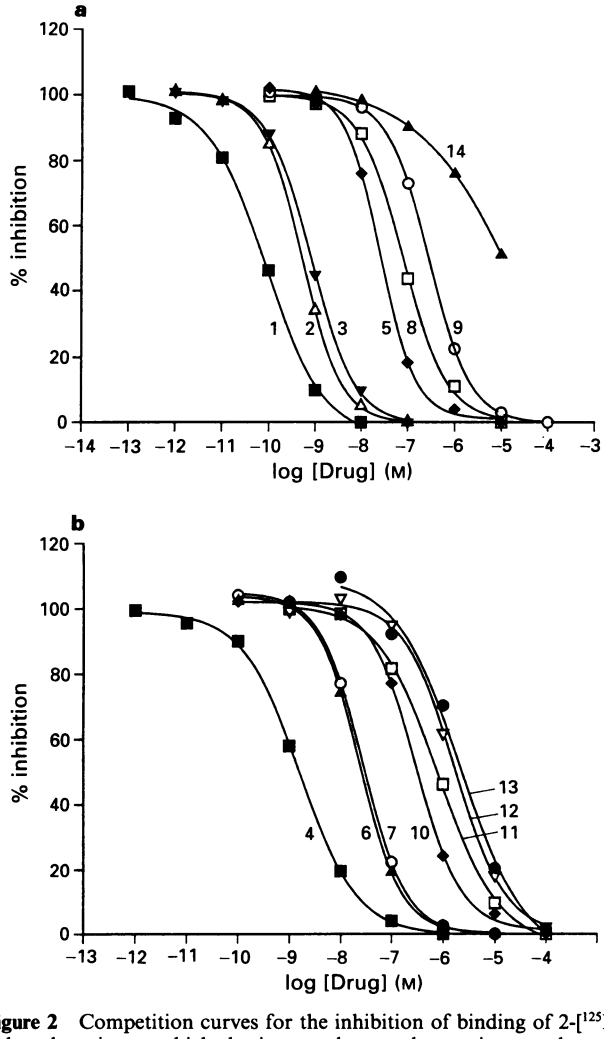


Figure 2 Competition curves for the inhibition of binding of 2-[^{125}I]-iodomelatonin to chick brain membranes by various melatonin analogues. (a) **1** 2-chloromelatonin; **2** melatonin; **3** 6-fluoromelatonin; **5** 6-hydroxymelatonin; **8** 6-methoxymelatonin; **9** 5-methyl *N*-acetyltryptamine; **14** 5-methoxytryptamine. (b) **4** 6-Chloromelatonin; **6** N-isobutyanoyl 5-methoxytryptamine; **7** N-valeroyl 5-methoxytryptamine; **10** 5-benzoyl *N*-acetyltryptamine; **11** O-acetyl 5-methoxytryptamine; **12** N-acetyltryptamine; **13** N-acetyl 5-hydroxytryptamine. The data shown are the mean of duplicate determinations from a representative experiment.

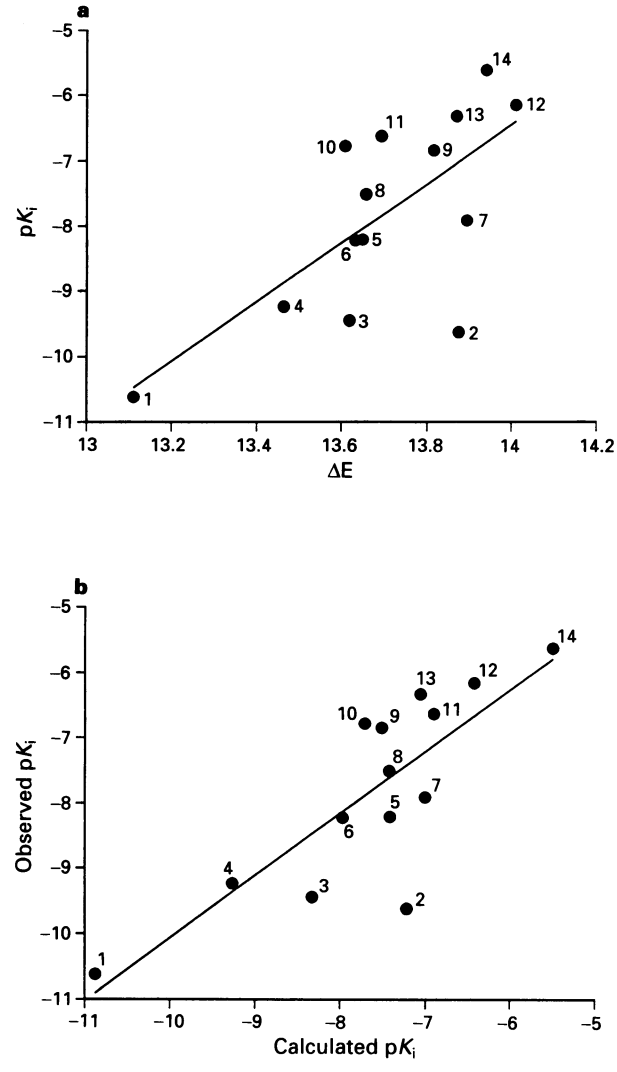


Figure 3 (a) Correlation of ΔE and inhibition constants (pK_i) for melatonin analogues for the displacement of 2-[^{125}I]-iodomelatonin binding from chick brain membranes. Compound numbers are given in the legend to Figure 2. (b) Graph of experimentally observed pK_i against calculated pK_i. Calculated pK_i values were obtained using the equation $pK_i = -4.9\ \Delta E + 45.3\ Q_NH + 64.8$, except for melatonin (**2**) which was determined from the equation $pK_i = 770.7\ \Delta E^{-1} + 51.2\ Q_NH - 58.8$.

Discussion

Our previous studies (Sugden & Chong, 1991) have shown that the binding site identified by 2-[^{125}I]-iodomelatonin in chick brain membranes is pharmacologically identical to the melatonin receptor in sheep pars tuberalis, at least as defined using a number of tryptamine analogues of melatonin including those used in the present analysis. Activation of melatonin receptors on pars tuberalis cells has been shown to inhibit forskolin stimulation of adenosine 3':5'-cyclic monophosphate (cyclic AMP) production (Morgan *et al.*, 1989), and it has been proposed that the pars tuberalis may be the site of action of melatonin in mediating photoperiodic changes in reproductive status (for discussion see Morgan *et al.*, 1994). The chick brain site also has very similar binding characteristics and pharmacology to the receptor in chick and rabbit neuronal retina which regulates dopamine release from amacrine cells (Dubocovich, 1985). In chick brain membranes, GTP inhibits 2-[^{125}I]-iodomelatonin binding suggesting that the binding site is coupled to a guanine nucleotide binding protein (G-protein) and thus is a functional receptor linked to an intracellular second messenger system. In some other tissues, for example, neonatal pituitary, pars tuberalis and *Xenopus laevis* melanophores, it has been shown that the melatonin receptor is negatively coupled to adenylate cyclase through a G_i-like protein as pertussis toxin blocks the biochemical effects or biological action of melatonin (White *et al.*, 1987; Vanecek & Vollrath, 1989; Carlson *et al.*, 1989; Sugden, 1991). Coupling to other transduction systems has also been reported (Vanecek & Vollrath, 1990; Vanecek & Klein, 1992). Using an expression cloning strategy, a melatonin receptor has recently been isolated from *Xenopus laevis* melanophores (Ebisawa *et al.*, 1994). The cDNA encodes a protein of 420 amino acids which has similarities to a wide range of G protein-coupled receptors rather than to any one particular group. Nevertheless, the melatonin receptor sequence contains seven hydrophobic segments which, like other G protein-coupled receptors, are thought to fold into transmembrane α-helical domains.

Existing receptor binding site models for adrenoceptors, dopamine, muscarinic and 5-HT_{2c} receptors have been devised from site-directed mutagenesis studies and the clear homology with the transmembrane domains of bacteriorhodopsin (Strader *et al.*, 1987; Hibert *et al.*, 1991; Lewis *et al.*, 1993). These models suggest that four or five of the transmembrane helical segments may be involved in ligand binding, and that a conserved aspartate on helix III and a serine or threonine residue on helix V are important for ligand binding. In the 5-HT_{2c} receptor site model (Lewis *et al.*, 1993) an alignment of helices II–VI in an approximately parallel symmetric arrangement gave rise to an interhelical channel of appropriate dimensions for binding of 5-HT. The putative binding site lies near the top of this channel and contains an aspartate and serine residue which pair with the protonated amino group and act as a hydrogen bond donor/acceptor with the indole 5-hydroxy group respectively. Two aromatic amino acid residues (phenylalanine and tryptophan) are found in transmembrane helix VI in the rat 5-HT_{2c} receptor which may enter into π–π stacking interactions with the indole ring of 5-HT (Lewis *et al.*, 1993). These residues are also found in the human β_1-adrenoceptor and muscarinic receptors and in bovine rhodopsin where a similar interaction may occur with the aromatic rings of the respective ligands.

An examination of the quantitative structure-affinity relationships for the melatonin analogues investigated and the similarity of the structures of 5-HT and melatonin, suggest that the amide moiety of melatonin may participate in hydrogen bonding. This is indicated by the appearance of the highest occupied molecular orbital frontier electron population on the amide nitrogen of the side-chain (Q_NH) in the QSAR equations which suggests that this group might act as a proton donor in a cooperative charge transfer interaction, assisted by the electronic donor-acceptor characteristics of the indole nucleus. Furthermore, the appearance of ΔE in the QSAR equations may reflect π–π stacking between the indole nucleus of the melatonin derivatives and an aromatic amino acid residue in the receptor binding site. This type of interaction between delocalized ring systems is assisted by frontier orbital (electron transfer) interactions between the two rings. Using the molecular electrostatic potential map of melatonin (Lewis *et al.*, 1990) and its similarity to 5-HT, and by modelling the putative transmembrane regions of the cloned melatonin receptor of *Xenopus* melanophores (Ebisawa *et al.*, 1994), it has been possible to construct a putative binding site model (Figure 4). In this model, it is envisaged that hydrogen bonding can occur between the 5-methoxy and amide groups of melatonin and complementary Ser_{115} and Asn_{167} residues in putative transmembrane helices III and IV respectively. The binding site model proposes that the single Trp_{256} residue in helix VI overlaps with the indole ring of melatonin allowing charge transfer between receptor and ligand as suggested by the appearance of ΔE in the QSAR correlations. Melatonin can be docked into this postulated binding domain in at least two ways such that hydrogen bonding can occur. The model shown (Figure 4) seems most probable in terms of the likely interactions between melatonin and complementary amino acid residues, namely the indole ring of melatonin and Trp_{256} via π–π stacking, hydrogen bonding between the -NH moiety of the melatonin side-chain and Asn_{167}, and Ser_{115} donating a hydrogen bond to the $-CH_3O$ moiety of melatonin. In addition, hydrophobic interactions between the three non-polar residues (Ile_{89}, Val_{170}, and Ile_{194}) and the methoxymethyl group at the 5-position, the amide methyl group and methylene side-chain of melatonin respectively are likely. From the model it appears that there is space for both an increase in the size of the amide side-chain and substitutions at the 2- and 6-position of the indole ring; such substitutions have been shown to be well-tolerated (Sugden & Chong, 1991).

The proposed model suggests that sufficient room exists for a water molecule between melatonin and the receptor, and does not preclude the possibility that hydrogen bonds may form either between the ligand and receptor or between the ligand and water molecule and the receptor. This is interesting in view of the recent observation that the van't Hoff plot of affinity against temperature for 2-[^{125}I]-iodomelatonin

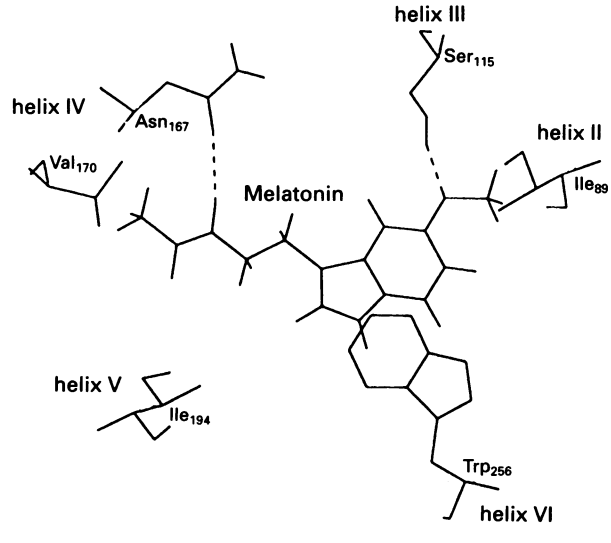


Figure 4 The postulated melatonin receptor binding site model. This model shows two possible hydrogen bonded interactions between melatonin and serine and asparagine residues in transmembrane helices III and IV of the receptor. Potential hydrogen bonds are denoted by dashed lines.

binding in chick brain membranes is not linear (Chong & Sugden, 1994). The fundamental properties of water molecules suggest that the ease of hydrogen bond formation between the ligand, water and the receptor would vary with temperature.

Assuming two hydrogen bonds are formed the thermodynamic binding energies for melatonin can be calculated using the equation of Williams *et al.* (1991):

$$\Delta G_{binding} = \Delta G_{T+R} + \Delta G_{Rotors} + \Delta G_{Hyd} + \Delta G_{Polar}$$

where $\Delta G_{binding}$ is the overall free energy of binding, ΔG_{T+R} is the loss in free energy due to restricted transition and rotation of the molecule, ΔG_{Rotors} is the loss in free energy due to loss in internal rotational freedom of associating components during binding, ΔG_{Hyd} is the favourable free energy change accompanying the association of hydrophobic species caused by desolvation, ΔG_{Polar} is the favourable free energy change due to interactions between polar functional groups, such as hydrogen bonding.

For melatonin the magnitude of each contribution to ΔG can be calculated from published data as follows: $\Delta G_{T+R} = 56$ kJ mol^{-1} for a molecule of mass 232 daltons binding to a macromolecule in an aqueous environment, $\Delta G_{Rotors} = 4-6$ kJ mol^{-1} for substrates with a relative molecular mass of a few hundred daltons, $\Delta G_{Hyd} = -0.19$ kJ mol^{-1} per A^2 of surface removed from exposure to water on binding. The surface area of melatonin is 488.2 A^2, ΔG_{Polar} = intrinsic binding energy of functional groups which would be between 12.552–16.736 kJ mol^{-1} per hydrogen-bond (as the energy varies according to the nature of the electronegative atoms involved in the hydrogen bond). Taking the averages for these contributions and assuming two hydrogen bonds are formed between the amide and 5-methoxy groups of melatonin and the receptor, $\Delta G_{binding} = 56 + 5 - 92.758 - 2(14.644) = -61.046$ kJ mol^{-1}.

Using the maximum and minimum values for ΔG_{Polar} and ΔG_{Rotors}, $\Delta G_{binding}$ varies between -57.86 and -64.23 kJ mol^{-1}. This calculation gives a value which agrees extremely well with experimentally determined value, predicting a K_i between 16 and 185 pM at 25°C. The experimentally determined K_i for melatonin at the chicken brain receptor at 25°C is 240 pM.

Further experiments will allow the molecular model proposed to be critically tested. First, a resurgence of interest in the pharmacology of melatonin has led to the synthesis and testing of novel analogues of melatonin by several groups. In particular, several novel series of potent melatonin analogues based on either a tetraline nucleus (Copinga *et al.*, 1993) or a naphthalene nucleus (Yous *et al.*, 1992) have recently been described. In addition, we have synthesized a series of high-affinity, conformationally restricted tetrahydrocarbazole analogues of melatonin (Garratt *et al.*, 1994) in which the amide side-chain has limited flexibility. It will be interesting to determine if these analogues can be docked into the proposed melatonin binding site. Second, the isolation of the cDNA for the melanophore melatonin receptor will facilitate the cloning of the mammalian melatonin receptor mediating the effects of the hormone on circadian and seasonal rhythms, and the identification of potential melatonin receptor subtypes (Krause & Dubocovich, 1991). Furthermore, site-directed mutagenesis studies will allow substitution or deletion of key amino acids enabling a direct test of the binding site model proposed.

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